



METHODS ARTICLE

Combining tangential flow filtration and size fractionation of mesocosm water as a method for the investigation of waterborne coral diseases

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Abstract

The causative agents of most coral diseases today remain unknown, complicating disease response and restoration efforts. Pathogen identifications can be hampered by complex microbial communities naturally associated with corals and seawater, which create complicating “background noise” that can potentially obscure a pathogen’s signal. Here, we outline an approach to investigate waterborne coral diseases that use a combination of coral mesocosms, tangential flow filtration, and size fractionation to reduce the impact of this background microbial diversity, compensate for unknown infectious dose, and further narrow the suspect pool of potential pathogens. As proof of concept, we use this method to compare the bacterial communities shed into six *Montastraea cavernosa* coral mesocosms and demonstrate this method effectively detects differences between diseased and healthy coral colonies. We found several amplicon sequence variants (ASVs) in the diseased mesocosms that represented 100% matches with ASVs identified in prior studies of diseased coral tissue, further illustrating the effectiveness of our approach. Our described method is an effective alternative to using coral tissue or mucus to investigate waterborne coral diseases of unknown etiology and can help more quickly narrow the pool of possible pathogens to better aid in disease response efforts. Additionally, this versatile method can be easily adapted to characterize either the entire microbial community associated with a coral or target-specific microbial groups, making it a beneficial approach regardless of whether a causative agent is suspected or is completely unknown.

Keywords: coral; disease; microbes; bacteria; SCTLD; water-borne

Introduction

The precarious plight of coral reefs today is well-established, as corals continue to decline in response to pollution, overfishing, ocean acidification, and global climate change [1, 2]. Over the last 50 years, an additional stressor has increasingly contributed to coral decline: the growing incidence and

prevalence of coral diseases [3, 4]. Culture-independent techniques such as genetic sequencing have considerably advanced our knowledge of coral diseases, but have also revealed the huge diversity and variability of coral microbiomes [5]. These complex microbial communities can make it difficult to distinguish the pathogen(s) from the

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“background noise,” and consequently the causative agents of most coral diseases remain unknown [6, 7]. Further complicating causative agent identification is the fact that some pathogens do not require large numbers to cause disease; within the better-studied human system for example, the bacterium *Francisella tularensis* is capable of inducing a disease response with exposure to a single bacterium [8], underscoring the importance of considering rare and abundant microbes equally in disease etiology investigations.

For waterborne coral diseases, the causative agent should be detectable within the seawater surrounding an infected colony. By characterizing the microbial communities of seawater associated with a diseased coral, some of the complicating background noise associated with the microbial communities within the mucus or tissue can be eliminated, as studies comparing bacterial species richness between coral tissue and surrounding seawater have found lower numbers of operational taxonomic units (a proxy for bacterial species) in seawater [9, 10]. Additionally, investigation of microbes shed by corals into seawater can help eliminate confounding findings within coral mucus or tissue, such as those associated with opportunistic secondary infections [11], because the primary pathogen potentially could be detected in the seawater itself before the onset of disease in surrounding apparently healthy colonies.

Here, we describe a method that combines screening coral mesocosm water via tangential flow filtration (TFF), followed by sequential sterile size fractionation, to investigate the etiology of waterborne coral diseases. Following this method, coral colonies are first isolated in individual mesocosms containing UV-treated and filtered seawater, and then incubated to allow for the mesocosms to become enriched with coral-shed microbes. TFF is then employed to concentrate the microbial communities in the coral mesocosms to ensure potentially important rare microbes are also detected. Finally, this TFF-concentrated mesocosm microbial community is filtered through a series of filters with different pore sizes to physically separate the concentrated microbial community into different size fractions that can be individually assayed. This novel combination of steps addresses the issues of (i) reducing the background noise of microbial diversity in coral tissues and seawater by focusing on a subset of transmissible microbes shed into mesocosms, (ii) concentrating that microbial community to maximize the chance of detecting even rare pathogens, and (iii) separating the concentrated microbial community into different fractions to expedite screening.

To demonstrate the utility of this method, we apply this approach to characterize microbial communities associated with stony coral tissue loss disease (SCTLD) [12]. SCTLD is an ideal system to demonstrate our method because: (1) it exhibits waterborne transmission [13–15], indicating the causative agent likely will be shed into the mesocosms and (2) several studies of infected coral tissue exist, allowing for comparison of our results to those obtained using more traditional approaches. To date all studies characterizing SCTLD-associated microbes through genetic sequencing have examined bacterial communities [16–21]. Consequently, as proof of concept of the practical application of this method, the exploratory pilot dataset presented here characterizes the bacterial size fraction of disease-associated coral mesocosms and we compare these results to results from prior SCTLD studies. Additionally, we also present a flexible experimental design that allows this approach to be applied to investigations of other coral-associated microbial groups.

Materials and methods

The methodology described below was developed over two time periods (October 2019 and November 2020), with slight modifications due to emerging knowledge of SCTLD.

Donor coral collection

“Donor” corals, or the corals contributing microorganisms into the mesocosm seawater via shedding, were collected from Florida reefs under permit FKNMS-2017-128-A2 by SCUBA divers using hammers and chisels. Colonies selected for collection were located at least 5 m apart to reduce the likelihood of sampling clones and whole and partial colonies were obtained. Apparently healthy individuals (i.e. individuals displaying no SCTLD signs) were collected in April 2018 ($n=1$) and January 2019 ($n=1$) from the Key West Nursery. Diseased individuals ($n=4$), characterized as those exhibiting SCTLD signs (tissue loss with or without bleaching along the margin [12]), were collected in the Florida Keys in October 2019 from offshore reefs near Summerland Key, FL ($n=2$) and November 2020 from reefs near Marathon, FL ($n=2$). To control for potential species-level differences in microbial community structure and composition, all corals utilized in this study ($n=6$) were *Montastraea cavernosa*.

Following collection, all corals were transported back to the shore in buckets or coolers, wrapped in bubble wrap moistened with seawater, and driven in coolers to the Smithsonian Marine Station in Ft. Pierce, FL. Upon arrival, apparently healthy corals were rinsed with filtered seawater and placed into a temperature-controlled indoor tank containing ~570 L of recirculating filtered seawater maintained at ~25.5°C and also housing other healthy corals. To ensure the healthy control corals were naïve to SCTLD, both healthy colonies were held in this tank from the time of collection until the start of our experiment (a minimum of nine months). Additional details on this system are described by Ushijima et al. [20]. Diseased corals were also rinsed with filtered seawater upon arrival at the Smithsonian and visible epibionts were removed by hand. Diseased colonies were then placed immediately into individual mesocosms, as described below.

Mesocosm creation

Mesocosms were created by filling ~19 L buckets with ~18 L of seawater that had been UV-treated and 0.22 µm filtered to reduce the starting microbial load. These buckets were placed in outdoor water tables maintained at ~28°C and located under a mesh canopy to allow some sunlight attenuation. Additional details on the seawater system and facilities are described by Ushijima et al. [20]. Each bucket mesocosm received a donor coral and an airstone to circulate and oxygenate the water. While mesocosms remained isolated from each other within the water tables, separate water tables were used for diseased and healthy mesocosms to further reduce potential cross-contamination.

To enrich the mesocosms with coral-shed microbiota, diseased donor corals were incubated within these mesocosms for 3–4 days, which was the maximum amount of time diseased corals could remain in the mesocosms without water changes before water quality deteriorated considerably due to sloughing tissue. Healthy donor corals were also incubated for 3–4 days for consistency. However, SCTLD exhibits particularly rapid lesion progression [12]; for other, more slowly progressing diseases,

additional incubation time may be possible to further enrich the mesocosm water with coral-shed microbes.

Tangential flow filtration

Following incubation, the seawater from each mesocosm was first pre-filtered through an ethanol-sterilized 200 μm (October 2019) or 106 μm (November 2020) mesh screen to remove particulate matter. Although the two different sizes employed here were due to opportunistic availability and both effectively trapped coral-shed particulates, we recommend using the finer mesh size (106 μm) at this stage to ensure efficient removal of particulates that may clog the subsequent filtration stages. This pre-filtered water was then pumped via peristaltic pump through a TFF manifold (Pall Corporation, #FS012K10) using Masterflex I/P Precision Pump Tubing, platinum-cured silicone, I/P 26 (Cole Parmer, #96410-26) and I/P 73 (Cole Parmer, #96410-73) and containing five 100 kDa Omega Centramate filter cassettes (Pall Corporation, #OS100T12) to concentrate the microbial community into $\leq 250\text{ mL}$ volume [22]. The 100 kDa pore size was employed here to effectively capture all bacterial and viral components of the mesocosm microbial communities [22], however, other pore size membranes could be substituted to address different research questions. The complete TFF concentration process is depicted in Fig. 1. Between mesocosm samples, the TFF manifold was flushed with reverse osmosis (RO) water to flush out and dilute any residual microbes. Healthy samples were processed first and then the system was flushed with a $\sim 0.04\%$ v/v sodium hypochlorite (bleach) solution (40 mL of $\sim 5\%$ sodium hypochlorite in 5 L RO water) and RO water before processing diseased samples. Following the October 2019 processing run, the system was flushed with bleach solution for 1 h, RO water for 2 min, and 0.1 N NaOH solution for

5 min prior to disassembly and storage. Filter cassettes were stored in NaOH at 4°C between October 2019 and November 2020. Prior to beginning processing in November 2020, the system was flushed with deionized water for 30 min.

Size fractionation

Following TFF concentration, each TFF-concentrated sample was filtered through a sequential series of filters with different pore sizes to partition each mesocosm microbial community into different-sized components. In October 2019, the TFF-concentrated microbial community was directly filtered through a Corning sterile filter unit containing a $\sim 47\text{ mm}$ 0.22 μm nitrocellulose membrane (#430756) to capture all microorganisms larger than 0.22 μm , which includes microeukaryotes, most bacteria, and large viruses. This filter was subsequently cut from the unit using a sterile razor blade. The resulting 0.22 μm filtrate from each filter unit, containing microbes smaller than 0.22 μm (most viruses), was then drawn through an autoclave-sterilized Millipore 47 mm mixed ester 0.025 μm filter (#VSWP04700) housed within an autoclave-sterilized filter funnel similar to 300 mL, 47 mm diameter Pall magnetic filter funnels (Pall Corporation, #4242) on a filter manifold similar to Fisherbrand PVC vacuum manifold (Fisher Scientific, #09-753-39A). This “viral” filtration was terminated after $\sim 5\text{ h}$ due to time constraints. All filters were preserved at -20°C until processing.

Following the October 2019 processing run, findings were published suggesting a possible bacterial causative agent for the disease [13]. Thus, here we characterize the microbial communities retained on the 0.22- μm filters as a proof of concept of this methodology. However, given that a benefit of this method is the flexibility offered by the optional addition of more filter

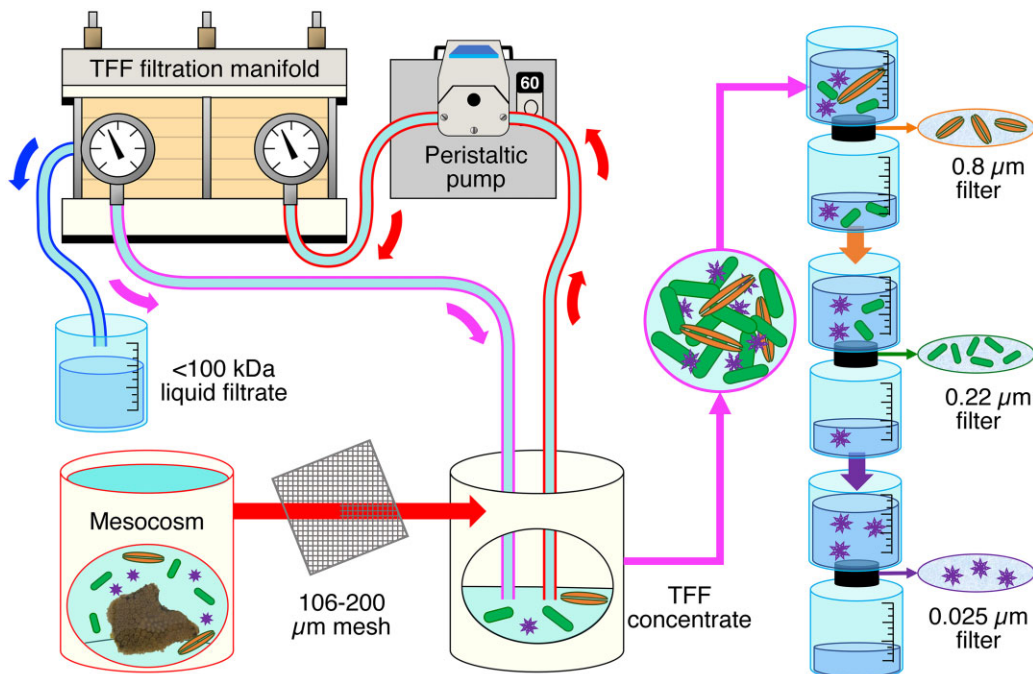


Figure 1: Flow chart depicting our methodology for investigating waterborne coral disease. Corals are first incubated in mesocosms (in red) containing filtered/UV-treated seawater to enrich the system with shed microbes, including the unknown pathogen. Mesocosm water is pre-filtered through a mesh screen to remove sloughed tissue, mucus, and other debris. Mesocosm water (in red) is then pumped via peristaltic pump through a TFF manifold containing 100 kDa filter cassettes, with anything smaller than 100 kDa passing out as filtrate (in blue) and anything larger than 100 kDa (in pink) recirculated back into the system. The concentrated microbial community, or “TFF concentrate” (in pink), is then passed through a series of 0.8 μm (in orange), 0.22 μm (in green), and 0.025 μm (in purple) filters to size fractionate the microbial community. The filter sizes utilized here can be removed or substituted with other filter sizes as appropriate for the research question.

sizes, we needed to confirm that such manipulation would not influence our ability to characterize the microbial community of subsequent smaller filtration stages. Therefore, in November 2020, two additional diseased corals and the same two healthy corals used in October 2019 were incubated in mesocosms and processed as described above, with the addition of a sterile Nalgene filter unit containing a 50 mm 0.8 μ m cellulose nitrate membrane (#126-0080) prior to 0.22 μ m filtration. This addition was intended to separately isolate the microeukaryote size fraction from the bacterial component. The complete size fractionation process is depicted in Fig. 1.

Epifluorescence microscopy

In order to demonstrate that all components of this experimental design function as expected, 3 mL water samples for visualization with epifluorescence microscopy were acquired at each experimental stage: the starting 0.22 μ m-filtered and UV-treated seawater (November 2020 only), the coral mesocosm post-incubation, the TFF concentrate, the 0.8 μ m filtrate (November 2020 only), and the 0.22 μ m filtrate. From each 3 mL sample, 1 mL was filtered in duplicate onto 25 mm diameter, 0.02 μ m Whatman Anodisc filters (Cytiva, #68096002) on a Sartorius glass frit filtration holder (Sartorius, #16306), and a side-arm flask plumbed to a vacuum pump. Filters were stained with SYBR Gold (Invitrogen, #S11494) based on a modified version of the SYBR Green protocol described by Noble and Fuhrman [23]. Filters were applied to glass microscope slides and stored at -20°C until visualization. Visualization was performed with an Olympus BX51TRF fluorescence light microscope equipped with a filter cube for SYBR Gold and images were acquired using an Olympus DP22 camera and Olympus cellSens Standard imaging software v.3.1. Although not essential for the utility of this method overall, we recommend completing this visualization stage as a quality control check.

DNA extraction

DNA was extracted from half of each 0.22 μ m filter using a DNeasy PowerBiofilm kit (Qiagen, #24000-50), following the manufacturer's standard protocol (QuickStart Protocol, November 2016 version), except for a substitution at stage 5 for bead beating at 2500 rpm for 30 s. A kit blank containing no filter was processed simultaneously and a positive control [MSA-3001 ABRF-MGRG 10 Strain Even Mix Genomic Material (ATCC, #MSA-3001)] was included for all subsequent processing stages. DNA quantification on a Qubit Fluorometer revealed that DNA extraction was not successful for the November 2020 healthy samples, so these samples were removed and only the remaining eight samples were processed further.

Amplicon library preparation and sequencing

Amplicon libraries were prepared by the RTSF Genomics Core at Michigan State University following the dual-index sequencing strategy developed by Kozich et al. [24] and using the primers 515F (5'-GTG CCA GCM GCC GCG GTA A-3') and 806R (5'-GGA CTA CHV GGG TWT CTA AT-3') [25]. Variations on these primers that allow for the enhanced detection of specific bacterial and archaeal taxa are available (e.g. [26, 27]) and researchers should accordingly consider their research question and compare primer capabilities before sequencing. Batch normalization was performed with Invitrogen SequelPrep DNA Normalization plates. Subsequent products were pooled, concentrated in an Amicon spin column, and purified with AMPure XP magnetic

SPRI beads. DNA sequencing was performed on an Illumina MiSeq v2 Nano flow cell in a 2 \times 250 bp paired end format, with base calling conducted via Illumina Real Time Analysis v1.18.54 and subsequent demultiplexing performed with Illumina Bcl2fastq v2.20.0. Raw sequence data were deposited online through NCBI (BioProject PRJNA731170) and in a U.S. Geological Survey data repository, available at <https://doi.org/10.5066/P9B13K8N> [28].

Bioinformatic processing

Demultiplexed sequences were imported into QIIME2 v2021.4 [29]. The DADA2 [30] plugin was used under default parameters to denoise sequences, remove chimeric sequences, and group sequences into amplicon sequence variants (ASVs), with sequences left-trimmed at position 13 and truncated at position 200. Sequences were aligned and a phylogenetic tree was constructed using MAFFT v7.0 [31] and FastTree 2 [32]. Taxonomy was assigned using the pre-trained naïve Bayes classifier SILVA-138-99-515-806 [33] and sequences matching chloroplasts or mitochondria were removed.

Positive and negative controls were removed from the data set and remaining samples were rarefied to 88 204 sequences to standardize the sampling depth across samples. Alpha and beta diversity analyses were conducted using the q2-diversity plugin and the "core-metrics-phylogenetic" approach within QIIME2, and revealed that the sample size used in this exploratory dataset was too small to statistically assess relationships between the different samples. Instead, a principal coordinates analysis (PCoA) based on a weighted UniFrac distance matrix was performed and an Emperor PCoA plot was generated to visualize the relationships between different samples.

Results comparison

To assess the utility of our methodology in characterizing coral microbiomes, the NCBI BLASTn sequence alignment service was used to directly compare ASVs obtained from our exploratory dataset to ASVs indicated as enriched in SCTLD-affected coral tissue samples within prior studies [16–18]. ASV-level analyses were conducted based on our unrarefied dataset and comparison ASVs were acquired from publicly available repositories. Due to variations in sequence length/areas of overlap, 100% query cover was not possible, so ASVs were considered to be the same as those identified in prior studies based on 100% sequence identity match and a high percentage query cover (>85%).

Results

Methodology

Using the methodology described herein (Fig. 1), mesocosm microbial communities were concentrated from \sim 18 L to between \sim 190 and 250 mL. Epifluorescence microscopy images demonstrate that all stages of this experimental design perform as expected (Fig. 2): the corals shed microbes into the mesocosm water (Fig. 2A), the TFF process concentrates these microbes (Fig. 2B), and the subsequent size fractionation filtration stages partition the expected size classes of microbes (Fig. 2C, D). We also confirmed that the microbial load present within the filtered and UV-treated seawater initially used within the mesocosms was comparatively low (Fig. 2E).

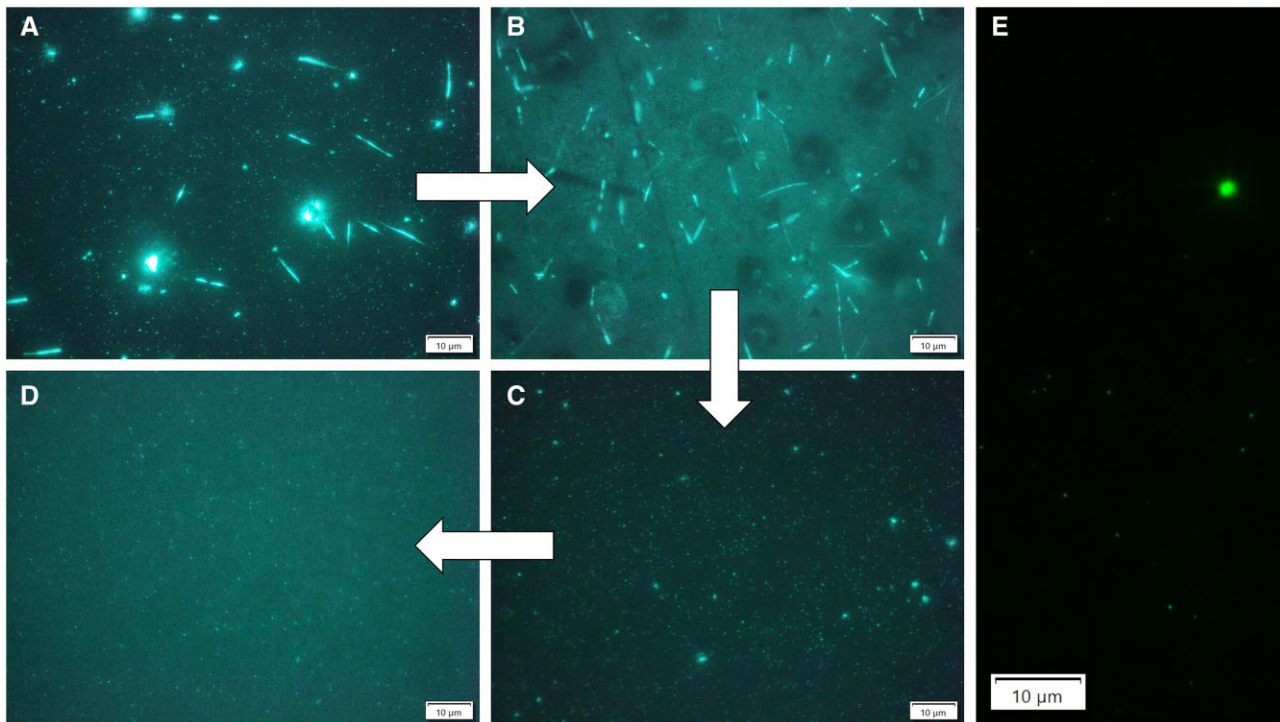


Figure 2: Example epifluorescence microscopy images, indicating success of TFF in concentrating mesocosm microbial communities and demonstrating the utility of size fractionation in removing different size classes of the microbial community. Example images are from a single diseased coral mesocosm (McD-58) from the November 2020 run and include samples from the initial mesocosm (A), post-TFF concentration (B), post-0.8 μm filtration (C), and post-0.22 μm filtration (D). Also shown is a sample of the 0.22 μm -filtered/UV-treated seawater used in the initial mesocosms (E), demonstrating the reduced microbial load of this water. Rod-shaped structures represent probable diatoms (A and B), larger dots are bacteria (A–C, E), and smaller dots are viruses (A–E). Photographs by J.S. Evans.

Exploratory dataset

In total, 800 992 raw sequences were obtained from the eight samples in the pilot dataset presented here, with 708 783 corresponding to the six coral mesocosms, 92 074 to the positive control, and 135 to the negative control. Of the mesocosm-derived sequences, 602 624 sequences representing 2837 unique ASVs remained following bioinformatic processing. On average, the numbers of sequences acquired for each sample type were relatively similar, with $\sim 97\,319$ sequences associated with October 2019 diseased mesocosms (max = 106 433, min = 88 204), $\sim 96\,993$ with November 2020 diseased mesocosms (max = 101 725, min = 92 260), and 107 001 with October 2019 healthy mesocosms (max = 115 976, min = 98 026). The weighted UniFrac Emperor PCoA plot revealed that microbial community structure varied between the different health states and experimental runs (Fig. 3), but these relationships were unable to be statistically analyzed due to the small sample sizes employed.

Becker et al. [16] identified 25 SCTLD “bioindicator” ASVs that were enriched within SCTLD diseased coral tissue compared with healthy corals. Comparing these 25 bioindicator ASVs against all coral mesocosms within our exploratory dataset, we identified 16 that were 100% sequence matches with ASVs in our exploratory dataset (Table 1). Becker et al. [16] further identified four bioindicator ASVs that were also 100% matches for SCTLD-associated ASVs from other studies ($n=2$, Meyer et al. [17] and $n=2$, Rosales et al. [18]). Using BLASTn, we confirmed that three of these four ASVs were present in our dataset (Table 1).

We further identified four ASVs within our exploratory dataset that were simultaneously SCTLD-obligate (present in all four diseased mesocosms) and SCTLD-specific (present exclusively

in diseased, and not healthy, mesocosms). Of these, two ASVs (ASV223, genus *Cohaesibacter* and ASV539, family *Arcobacteraceae*) were previously identified as SCTLD bioindicator ASVs [16] and one (ASV223, genus *Cohaesibacter*) was additionally identified as SCTLD-associated by another study [18] (Table 1). When the definition of “SCTLD-obligate” is expanded to include ASVs present in 75% of all diseased samples, an additional 31 ASVs qualify as “SCTLD-obligate-and-specific.” Two of these additional 31 ASVs (ASV141, genus *Fusibacter* and ASV312, genus *Vibrio*) are also 100% sequence matches for SCTLD bioindicator ASVs previously identified by Becker et al. [16], including one which was a 100% match for an ASV additionally identified as SCTLD-associated by Meyer et al. [17] (Table 1). A BLASTn comparison of Meyer et al.’s [17] ASV revealed a 100% sequence identity match with our ASV312 (genus *Vibrio*).

Discussion

Here, we have described a methodology for the investigation of waterborne coral diseases, characterizing the microbial community shed by a disease-infected coral into mesocosm seawater. Our exploratory results confirm that differences in the donor corals are discernible within the microbial communities shed by that coral, supporting the application of this method in coral microbiome investigations. However, it should be noted that while we were able to control for the influence of some potentially confounding variables (e.g. donor coral species), due to sampling limitations we were unable to eliminate all potentially confounding variability within the donor corals. For example, while health status of the donor coral may be contributing to some of the observed differences between samples, a possible

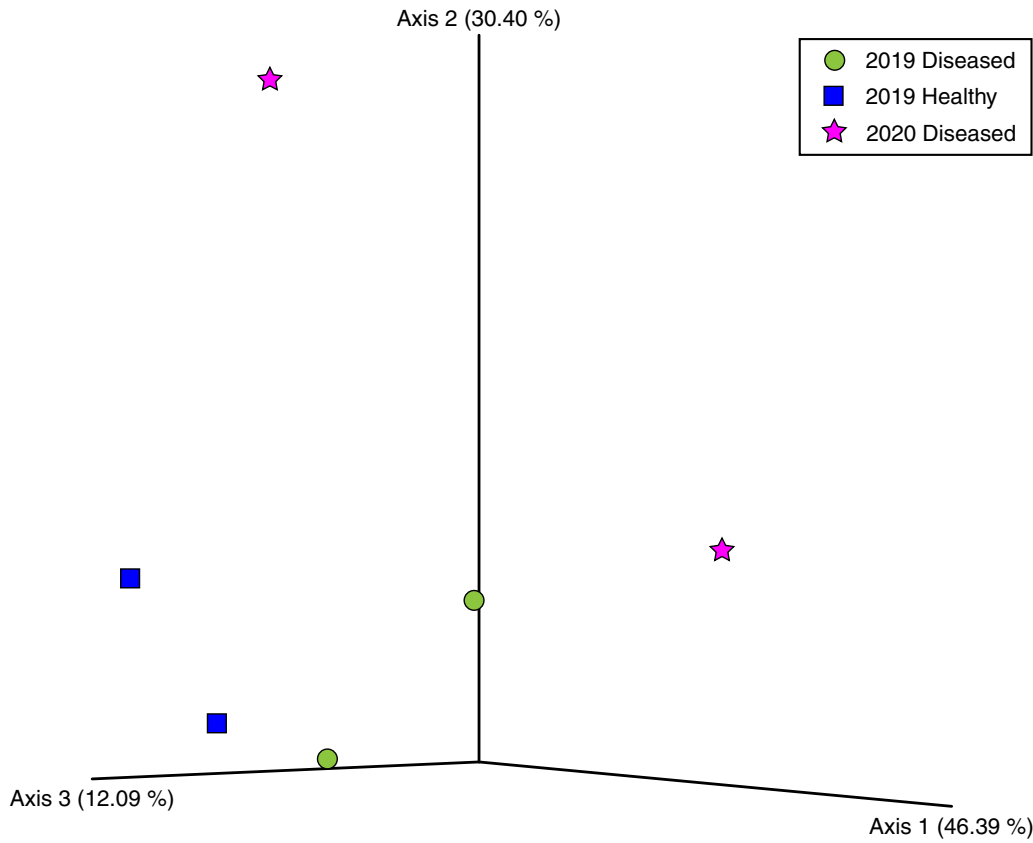


Figure 3: Weighted UniFrac Emperor PCoA plot depicting the relationships between the bacterial/archaeal communities associated with the six coral samples, obtained from 0.22 μm filters. October 2019 diseased samples are represented by green circles, November 2020 diseased samples represented by pink stars, and October 2019 healthy samples represented by blue squares. Axes percentages indicate the percentage variation in microbial community structure explained by that axis.

Table 1: ASVs acquired in our exploratory dataset compared with SCTLD bioindicator ASVs identified by Becker et al. [16]

ASV ID		Abundance			Taxonomy
Becker et al. [16]	This study	October 2019 diseased	November 2020 diseased	October 2019 healthy	Lowest classification
ASV13	ASV35	1728	919	223	<i>g. Halodesulfobivrio</i>
ASV20	ASV61	1064	16	544	<i>g. Vibrio</i>
ASV25	ASV325	0	0	135	<i>g. Vibrio</i>
ASV26	ASV739	0	36	0	<i>g. Uncultured Prolixibacter</i>
ASV36	ASV249	191	0	4	<i>s. Tepidibacter mesophilus</i>
ASV39	ASV244	0	203	0	<i>g. Marinifilum</i>
	ASV52	28	1876	0	<i>g. Marinifilum</i>
	ASV23	0	4835	0	<i>g. Marinifilum</i>
ASV44	ASV185	0	308	0	<i>g. Fusibacter</i>
ASV54 [*]	ASV312	6	134	0	<i>g. Vibrio</i>
ASV60	ASV66	55	9	1457	<i>g. Shimia</i>
ASV67	ASV268	58	0	115	<i>g. Vibrio</i>
ASV101	ASV539	34	29	0	f. Arcobacteraceae
ASV111 [†]	ASV440	0	88	0	<i>f. Rhodobacteraceae</i>
ASV135	ASV1301	13	0	0	<i>g. Fusibacter</i>
ASV226[†]	ASV223	112	124	0	g. Cohaesibacter
ASV263	ASV265	0	176	0	<i>f. Arcobacteraceae</i>
ASV275	ASV141	157	319	0	<i>g. Fusibacter</i>

Notes: All ASVs presented here exhibited 100% identity matches with 89% query cover. Becker et al.'s [16] ASV39 was 100% identical with 89% query cover to three ASVs in our dataset, likely due to sequence trimming differences, so all three possible matches are presented. Superscript characters denote bioindicator ASVs [16] that were also present in Meyer et al. (*) or Rosales et al. (†) studies [17, 18]. Gray shading highlights SCTLD-obligate-and-specific ASVs (found in at least 75% of diseased coral mesocosms and not in healthy coral mesocosms), and bold text indicates ASVs for which "obligate" included all diseased mesocosms. Abundance columns indicate the total reads for each ASV in October 2019 diseased, November 2020 diseased, and October 2019 healthy mesocosms. Lowest classification indicates the lowest taxonomic classification of each ASV, where f = family, g = genus, and s = species.

temporal effect on microbial community structure is also apparent (Fig. 3). Although we were unable to statistically evaluate these relationships, this suggests that numerous donor coral differences in addition to health state (e.g. collection date, source reef, year, and experimental run) may also influence coral-shed microbial communities and should be considered and minimized as much as possible in the experimental design stage. Nevertheless, here we also detected within our mesocosms several ASVs previously identified as SCTLD-associated, suggesting that donor coral health state may play an especially important role in shaping coral-shed mesocosm microbial communities, and further demonstrating the utility of our approach as a method for coral disease investigation.

Our exploratory results also indicated that while healthy mesocosm microbial communities appeared to cluster together, the microbial communities associated with SCTLD-diseased samples were much more scattered (Fig. 3). While this trend was unable to be confirmed statistically, this result is consistent with other investigations of microbial communities from SCTLD-diseased and apparently healthy corals [18], including for *M. cavernosa* in particular [16, 17]. Further, using this method, we successfully detected numerous ASVs previously indicated as SCTLD-associated within our mesocosms [16–18]. Interestingly, some ASVs previously noted as possible SCTLD bioindicators were detected in higher abundance in our healthy compared with diseased mesocosms (e.g. ASV66, genus *Shimia* and ASV268, genus *Vibrio*), highlighting the added potential benefit of using a multifaceted approach to investigate coral disease etiology in order to further narrow down possible pathogens.

The relatively even number of sequences obtained from diseased individuals across the two sampling runs (i.e. with and without the addition of a 0.8- μ m filtration step), as well as the detection of previously identified SCTLD-associated ASVs [16, 18] in all diseased mesocosms, indicate that incorporating an additional filtration step does not impact downstream filter utility. However, previous studies have shown that including a prefilter stage can result in significantly different bacterial community compositions between the prefilter and the downstream filter [34]. This could explain some of the variation observed in the November 2020 diseased samples compared with October 2019 (Fig. 3) and suggests the importance of carefully considering experimental design in selecting filter sizes. It may also be beneficial to include a control mesocosm containing only the filtered seawater. As shown in Fig. 2E, even the 0.22- μ m-filtered and UV-treated seawater is not completely sterile and some microbial communities can be expected to develop within this seawater during the incubation period [35]. Including a seawater-only mesocosm control and processing it using the same incubation/TFF/size fractionation method described here would provide a “background mesocosm” microbial community that could be subtracted from the coral mesocosm microbial community to remove additional background noise and assist in further narrowing down the pool of potential pathogens.

The flexibility of the method described herein is what makes it so useful for coral disease investigations. For example, recent electron microscopy work on SCTLD samples identified filamentous virus-like-particles that may be implicated in disease causation or progression [36]. The approach we describe herein could be easily modified through the use of different filter sizes and sequencing primers to specifically isolate and target these unknown viruses for further investigation. Additionally, our described methodology is useful in all coral disease investigations, regardless of current knowledge of the disease's etiology. If a particular size class/microbial group is suspected to be implicated in a disease pathology,

then a single membrane size could be utilized to specifically select for the target microbe group. If multiple size classes are suspected, then multiple filter sizes could be employed to investigate each microbial group independently through the use of targeted primers and reference databases. If the etiology is entirely unknown, this approach allows for the targeted investigation of all possible size classes of microorganisms either through shotgun sequencing or fractionation. Size fractionation approaches have also previously been used in transmission experiments to determine which size class/microbial group is responsible for a particular coral disease [37, 38], which represents another possible useful extension of this methodology.

Data availability statement

All sequence data generated by this project and associated metadata are publicly available through NCBI (BioProject PRJNA731170) and a U.S. Geological Survey data release [28], <https://doi.org/10.5066/P9B13K8N>.

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Author contributions

C.A.K. conceived the project idea and developed the methodology. J.S.E., V.J.P., B.U., and C.A.K. performed the experimental work and collected the data. J.S.E. performed the data analysis. J.S.E. and C.A.K. prepared the manuscript. All authors provided feedback and approved the final manuscript.

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Conflict of interest statement. None declared.

References

1. Pandolfi JM, Bradbury RH, Sala E et al. Global trajectories of the long-term decline of coral reef ecosystems. *Science* 2003; 301:955–8. doi:10.1126/science.1085706
2. Hughes TP, Barnes ML, Bellwood DR et al. Coral reefs in the Anthropocene. *Nature* 2017;546:82–90. doi:10.1038/nature22901
3. Bourne DG, Garren M, Work TM et al. Microbial disease and the coral holobiont. *Trends Microbiol* 2009;17:554–62. doi: 10.1016/j.tim.2009.09.004

4. Weil E, Rogers CS. Coral reef diseases in the Atlantic-Caribbean. In: Dubinsky Z, Stambler N, (eds), *Coral Reefs: An Ecosystem in Transition*. Dordrecht: Springer Netherlands, 2011, 465–91.
5. Huggett MJ, Apprill A. Coral microbiome database: Integration of sequences reveals high diversity and relatedness of coral-associated microbes. *Environ Microbiol Rep* 2019; 11:372–85. doi:10.1111/1758-2229.12686
6. Richardson LL. Coral diseases: What is really known? *Trends Ecol Evol* 1998;13:438–43. doi:10.1016/s0169-5347(98)01460-8
7. Rosenberg E, Koren O, Reshef L et al. The role of microorganisms in coral health, disease and evolution. *Nat Rev Microbiol* 2007;5:355–62. doi:10.1038/nrmicro1635
8. Jones RM, Nicas M, Hubbard A et al. The infectious dose of *Francisella tularensis* (Tularemia). *Appl Biosaf* 2005;10:227–39. doi:10.1177/153567600501000405
9. Sunagawa S, Woodley CM, Medina M. Threatened corals provide underexplored microbial habitats. *PLoS ONE* 2010;5: e9554. doi:10.1371/journal.pone.0009554
10. Hernández-Zulueta J, Araya R, Vargas-Ponce O et al. First deep screening of bacterial assemblages associated with corals of the Tropical Eastern Pacific. *FEMS Microbiol Ecol* 2016;92: fiw196. doi:10.1093/femsec/fiw196
11. Gignoux-Wolfsohn SA, Aronson FM, Vollmer SV. Complex interactions between potentially pathogenic, opportunistic, and resident bacteria emerge during infection on a reef-building coral. *FEMS Microbiol Ecol* 2017;93. doi:10.1093/femsec/fix080
12. NOAA. Case Definition: Stony Coral Tissue Loss Disease (SCTLD). Available online: <https://floridadep.gov/rcp/coral/documents/stony-coral-tissue-loss-disease-sctld-case-definition> (accessed on 17 November 2020).
13. Aeby GS, Ushijima B, Campbell JE et al. Pathogenesis of a tissue loss disease affecting multiple species of corals along the Florida reef tract. *Front Mar Sci* 2019;6. doi:10.3389/fmars.2019.00678
14. Dobbelaere T, Muller EM, Gramer LJ et al. Coupled epidemiological-hydrodynamic modeling to understand the spread of a deadly coral disease in Florida. *Front Mar Sci* 2020;7. doi: 10.3389/fmars.2020.591881
15. Muller EM, Sartor C, Alcaraz NI et al. Spatial epidemiology of the stony-coral-tissue-loss disease in Florida. *Front Mar Sci* 2020;7. doi:10.3389/fmars.2020.00163
16. Becker CC, Brandt M, Miller CA et al. Microbial bioindicators of stony coral tissue loss disease identified in corals and overlying waters using a rapid field-based sequencing approach. *Environ Microbiol* 2021. doi: 10.1111/1462-2920.15718
17. Meyer JL, Castellanos-Gell J, Aeby GS et al. Microbial community shifts associated with the ongoing stony coral tissue loss disease outbreak on the Florida reef tract. *Front Microbiol* 2019; 10. doi:10.3389/fmicb.2019.02244
18. Rosales SM, Clark AS, Huebner LK et al. *Rhodobacterales* and *Rhizobiales* are associated with stony coral tissue loss disease and its suspected sources of transmission. *Front Microbiol* 2020;11:681. doi:10.3389/fmicb.2020.00681
19. Iwanowicz DD, Schill WB, Woodley CM et al. Exploring the stony coral tissue loss disease bacterial pathobiome (Preprint). *bioRxiv* 2021. doi:10.1101/2020.05.27.120469
20. Ushijima B, Meyer JL, Thompson S et al. Disease diagnostics and potential coinfections by *Vibrio coralliilyticus* during an ongoing coral disease outbreak in Florida. *Front Microbiol* 2020; 11:569354. doi:10.3389/fmicb.2020.569354
21. Clark AS, Williams SD, Maxwell K et al. Characterization of the microbiome of corals with stony coral tissue loss disease along Florida's coral reef. *Microorganisms* 2021;9. doi: 10.3390/microorganisms9112181
22. Paul JH, Jiang SC, Rose JB. Concentration of viruses and dissolved DNA from aquatic environments by vortex flow filtration. *Appl Environ Microbiol* 1991;57:2197–204. doi: 10.1128/aem.57.8.2197-2204.1991
23. Noble RT, Fuhrman JA. Use of SYBR green I for rapid epifluorescence counts of marine viruses and bacteria. *Aquat Microb Ecol* 1998;14:113–8. doi:10.3354/ame014113
24. Kozich JJ, Westcott SL, Baxter NT et al. Development of a dual-index sequencing strategy and curation pipeline for analyzing amplicon sequence data on the MiSeq Illumina sequencing platform. *Appl Environ Microbiol* 2013;79:5112–20. doi: 10.1128/Aem.01043-13
25. Caporaso JG, Lauber CL, Walters WA et al. Global patterns of 16S rRNA diversity at a depth of millions of sequences per sample. *Proc Natl Acad Sci USA* 2011;108:4516–22. doi: 10.1073/pnas.1000080107
26. Apprill A, McNally S, Parsons R et al. Minor revision to V4 region SSU rRNA 806R gene primer greatly increases detection of SAR11 bacterioplankton. *Aquat Microb Ecol* 2015;75:129–37. doi:10.3354/ame01753
27. Parada AE, Needham DM, Fuhrman JA. Every base matters: Assessing small subunit rRNA primers for marine microbiomes with mock communities, time series and global field samples. *Environ Microbiol* 2016;18:1403–14. doi:10.1111/1462-2920.13023
28. Kellogg CA, Evans JS, Voelschow JJ. Bacterial communities shed by *Montastraea cavernosa* coral fragments into filtered seawater mesocosms—raw data: U.S. Geological Survey data release. 2021. doi:10.5066/P9B13K8N
29. Bolyen E, Rideout JR, Dillon MR et al. Reproducible, interactive, scalable and extensible microbiome data science using QIIME 2. *Nat Biotechnol* 2019;37:852–7. doi:10.1038/s41587-019-0209-9
30. Callahan BJ, McMurdie PJ, Rosen MJ et al. DADA2: high-resolution sample inference from Illumina amplicon data. *Nat Methods* 2016;13:581–3. doi:10.1038/nmeth.3869
31. Katoh K, Standley DM. MAFFT multiple sequence alignment software version 7: improvements in performance and usability. *Mol Biol Evol* 2013;30:772–80. doi:10.1093/molbev/mst010
32. Price MN, Dehal PS, Arkin AP. FastTree 2—approximately maximum-likelihood trees for large alignments. *PLoS ONE* 2010;5:e9490. doi:10.1371/journal.pone.0009490
33. Bokulich NA, Kaehler BD, Rideout JR et al. Optimizing taxonomic classification of marker-gene amplicon sequences with QIIME 2's q2-feature-classifier plugin. *Microbiome* 2018;6. doi:10.1186/s40168-018-0470-z
34. Byappanahalli MN, Nevers MB, Shively D et al. Influence of filter pore size on composition and relative abundance of bacterial communities and select host-specific MST markers in coastal waters of Southern Lake Michigan. *Front Microbiol* 2021;12:665664. doi:10.3389/fmicb.2021.665664
35. Rahav E, Ovadia G, Paytan A et al. Contribution of airborne microbes to bacterial production and N-2 fixation in seawater upon aerosol deposition. *Geophys Res Lett* 2016;43:719–27. doi: 10.1002/2015gl066898
36. Work TM, Weatherby TM, Landsberg JH et al. Viral-like particles are associated with endosymbiont pathology in Florida corals affected by stony coral tissue loss disease. *Front Mar Sci* 2021;8. doi:10.3389/fmars.2021.750658
37. Kline DI, Vollmer SV. White band disease (type I) of endangered Caribbean acroporid corals is caused by pathogenic bacteria. *Sci Rep* 2011;1:7. doi:10.1038/srep00007
38. Barash Y, Sulam R, Loya Y et al. Bacterial strain BA-3 and a filterable factor cause a white plague-like disease in corals from the Eilat coral reef. *Aquat Microb Ecol* 2005;40:183–9. doi: 10.3354/ame040183